SEDIMENTATION IN THE ANGLE CENTRIFUGE

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PLATES 1 TO 3

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Differential centrifugation in a high speed angle centrifuge has become a standard procedure for the purification and concentration of viruses and other biological materials of submicroscopic size. The degree of centrifugation necessary for a given material has generally been determined by trial, since experimental data have not been available which would allow a prediction, with any certainty, as to just how particles of known size would behave in an angle centrifuge. Experimental conditions in the angle centrifuge are quite different from those afforded by a sector-shaped ultracentrifuge cell of the Svedberg type (1), in which the side walls are directed toward the axis of rotation, so that a particle originally near any one of them continues its own radial migration parallel to the surface and without interference with its movement. If a general correlation could be established between sedimentation in the angle centrifuge and in the ultracentrifuge, which exhibits a discrete and measurable sedimentation boundary indicative of the particle size, it would then be possible not only to predict the behavior of a known material in the angle centrifuge but to estimate an unknown particle size from experimental data.

For reasons already cited, it has been impossible to interpret satisfactorily certain results obtained with angle centrifuges during the course of investigating several animal viruses in this laboratory. Among the primary observations have been the following: the presence of residual infectivity in the supernatant fluid of virus suspensions even after prolonged centrifugation (2); the variation in the degree of sedimentation obtained under identical experimental conditions with different concentrations of the same material (3); the different degrees of sedimentation accomplished with equal concentrations of the same material centrifuged under conditions which differed only in that one rotor was spun in a vacuum and the other in the open air. Among other puzzling observations which have awaited satisfactory explanation has been that of Claude (4), who reported the appearance of abnormally sharp boundaries of *Limulus* hemocyanin in a high speed electrically driven angle centrifuge spinning in the open air. It would be expected that the normal diffusion of particles during the long centrifugation period required would have caused pronounced blurring of the boundary.

With the view of clarifying conceptions of the process by which sedimenta-

tion is accomplished in angle centrifuges, a systematic investigation of the problem was undertaken with rotors spinning both in the open air (5) and in a vacuum (2). In order to simulate conditions encountered with biological agents of especial interest, *i.e.*, the smaller viruses, it was considered advantageous to use some readily accessible, homogeneous material having a comparable particle size.

Material and Methods

The material selected for use in all experiments was the hemocyanin from *Limulus polyphemus*. This large respiratory protein has a molecular weight of several millions (6) and, as has been observed during experiments to be described in a subsequent report, almost all of the protein exists in the form of a component having a sedimentation constant of about 57×10^{-13} cm./sec./unit field in an appropriate buffer solution of pH 6.9 or in the native serum when properly handled. For most of the work, the protein was first purified by several high and low speed centrifugations and then suspended in a buffer solution containing 1 per cent NaCl. In other cases, the native serum was only centrifuged several times at low speed to remove the jelly-like material and other coarse particles. It was then diluted to the required protein concentration by the addition of the buffer solution. Concentration, sedimentation rate, and the certainty of homogeneity were determined by analysis with a refractive index method (7) in the vacuum type ultracentrifuge of Bauer and Pickels (8). Experiments were performed with solutions having hemocyanin concentration ranging from 0.04 to 1.6 per cent.

Similar runs were made with two high speed angle centrifuges, one electrically driven and spinning in the open air (5) and the other driven by compressed air (2) and spinning in high vacuum. In both instances use was made of 20 cm. duralumin rotors which accommodated transparent celluloid tubes 9 cm. in length and 1.3 cm. in diameter (2). The angles of the tubes for the electrical and vacuum centrifuges were 40° and 35°, respectively.

In order that a direct comparison might be made between the behavior of the protein in the ultracentrifuge and in the angle centrifuges, the level of the solution in the celluloid tubes and in the ultracentrifuge cell was so adjusted that the meniscus during centrifugation would in every case be exactly the same distance from the axis of rotation, namely, 5.9 cm. (Text-fig. 2). Comparison runs were always performed at the same speed, namely, 16,200 R.P.M., and for the same "equivalent sedimentation time." Determination of the equivalent time involved allowances for the different effective times of acceleration and deceleration, corrections to compensate for the lowered viscosity of the solution in the slightly warmer electrical centrifuge, and adjustments for the slower sedimentation in the more concentrated preparations. With certain special exceptions, routine procedures were followed with the electrical and vacuum centrifuges of using normal acceleration times of about 3 minutes and 9 minutes, respectively, and deceleration times of 7 minutes and 9 minutes, respectively, with deceleration especially gradual just before stopping the centrifuge. Just after each tube was filled initially to the required level, a column of clear paraffin oil was carefully added above the aqueous surface to give the meniscus better definition, to prevent evaporation, and for other reasons explained below.

In the higher concentrations of hemocyanin, its blue color permitted the displacement of a sharp boundary in a transparent celluloid tube to be detected and measured visually after the tube had been carefully removed from the rotor and slowly oriented to an upright position. In order to study more exactly the distribution of concentration, particularly in the dilute solutions, all tubes were photographed on a specially fitted optical bench. To avoid severe refraction of light by the curved surface of the tube, it was almost completely immersed in water within a small chamber having two flat, parallel glass sides at right angles to the optical path. Light was permitted to pass through only a 0.5 cm. vertical section of the tube. A large condensing lens and a ground glass screen were employed to provide an even diffuse illumination over the full length of the tube. From a mercury arc, monochromatic ultraviolet light of 3650 Å, which is strongly absorbed by hemocyanin, was isolated by a filter of nickel oxide. A reprojection lens having a focal length of 50 cm. was used to minimize the errors of parallax.

The magnification in every case was adjusted to such a value that a direct comparison in terms of radial distance could be made between the photograph of the tube and an absorption photograph of the same material taken in the ultracentrifuge after an equivalent time of sedimentation. In other words, when the photographs were placed side by side with the menisci matching, any other two matching levels represented equal distances from the axis of rotation, considering the photograph of the tube to characterize conditions which existed along its axis just before the end of the run. This was nearly true, since the horizontal angle (with respect to axis of rotation) subtended by the tube was small, and although the meniscus or the thin layer of solution representing a sharp boundary was vertical and slightly curved in the centrifugal field, its intersection with the axis of the tube changed only slightly when it was reoriented to a horizontal position in the gravitational field. As illustrated in photographs presented in Fig. 1, the meniscus frequently failed, because of surface tension, to reorient itself into a horizontal position. Care was taken on this account to photograph tubes in a direction which was tangential with respect to their position in the centrifuge.

Several different photographic exposures were made for each tube. It was determined experimentally what exposures were necessary to produce equal intensities with a clear fluid when the Svedberg absorption method (1) was used in conjunction with the ultracentrifuge. Since a cell of 1 cm. thickness was employed, the total absorption by a given solution was roughly the same as when light was directed through the same material held in a tube. Light passing through the clear oil layer above the solution furnished a convenient reference for judging the presence of light-absorbing material in the supernatant fluid.

The standard columns of solution in both the electrical and vacuum angle centrifuges had lengths corresponding to a radial distance of about 3.4 cm. In addition, studies were made with shorter columns having a projected radial length of 1.35 cm., which corresponded to the standard column length employed in the analytical ultracentrifuge. These shorter columns (*viz. d*, Fig. 1) with menisci at the standard radial distance were prepared by first placing in the tube a proper amount of a heavy nonmiscible fluid, namely, bromobenzene. Also, since theoretical consideration has suggested the importance of density gradients within the solution in inhibiting convective disturbances, experiments were performed with long columns of solution in which

synthetic density gradients had been provided. The gradient was produced by first preparing two hemocyanin solutions of exactly the same composition (in terms of grams per liter of final solution) except that 8 to 10 per cent sucrose was incorporated in one. Enough of the latter was placed in the tube to form about half the required column. Above this was gently added the second solution to the correct level. A few vertical strokes with a glass rod partially mixed the two fluids, giving a rather uneven but usable gradient. In figuring the equivalent sedimentation time, allowance was made for the increased viscosity of the section through which the boundary migrated. The correction amounted to only a few per cent in all cases.

In order to conduct experiments in which the effects of the deceleration forces and of any possible thermally activated convection could be differentiated from those connected with the sedimentation process, it was necessary to prepare artificial sedimentation boundaries of hemocyanin (*viz. a*, Fig. 1). These were made with a clear buffer solution and a solution of hemocyanin against which it had been dialyzed to establish an equilibrium of the salts. The clear solution was placed in a celluloid tube, and the heavier solution was then slowly added through a long hollow needle extending to the bottom of the tube and connected to a syringe exhausted gradually by an electrically driven mechanical system (9). Since in most of the sedimentation work a centrifugation time equivalent to 135 minutes at 16,200 R.P.M. and room temperature was employed, the artificial boundaries were made up at distances from the meniscus corresponding to the radial level reached by the boundary in the ultracentrifuge after this time, namely, 6.9 cm. (or 1.0 cm. from the meniscus; *viz. l*, Fig. 1).

EXPERIMENTAL

It was found possible to make reasonably sharp artificial sedimentation boundaries by the method cited, especially with the higher concentrations of protein. It was shown experimentally that tubes containing the artificial boundaries could be subjected without appreciable effect to all the disturbing influences (except rotation of the centrifuge) encountered in handling those containing real, sedimented boundaries. Blurring of a boundary after the tube had stood for 135 minutes within a stationary rotor and had been subjected to two reorientations between the vertical and inclined positions was only slightly more than that to be expected by reason of the normal diffusion process (*viz. a*, Fig. 1).

To test for other disturbances not related to sedimentation, artificial boundaries which had stood for 135 minutes were run to the same speed of 16,200 R.P.M. used for the sedimentation experiments; they were then immediately decelerated according to normal procedure. Sedimentation under these conditions could not have been appreciable. Some of the results are illustrated in Fig. 1. With 0.8 per cent solutions, no alteration of the boundary was noted in either long or short columns run in the vacuum centrifuge (*viz. f*). In the electrical, the boundary remained sharp and was displaced away from the meniscus in short columns (*viz. d*). In long columns, it was displaced with considerable blurring toward the meniscus (*viz. e*). When concentrations of

0.2 per cent were used, mixing was complete with both long and short tubes in the electrical centrifuge (*viz. b*), while in the vacuum type the boundary was not completely eradicated but suffered a great deal of spreading, even into the region of the meniscus (*viz. c*). Taking the electrical centrifuge to and from a speed of only 1000 R.P.M. caused only partial mixing, roughly comparable to that in tube *c*, Fig. 1.

With one exception, runs in both angle centrifuges at 16,200 R.P.M. for an equivalent time of 135 minutes showed no detectable boundaries with a concentration of 0.04 per cent, although a faint one was visible in photographs taken with the ultracentrifuge. When a concentration gradient of sucrose was employed in connection with the vacuum centrifuge, a small gradation in the concentration of protein could be vaguely discerned. With the concentration increased to 0.12 per cent in the same centrifuge, better clearing (*viz. s*, Fig. 2) of the solution was obtained, without boundary formation. Boundaries were barely discernible with contents of 0.2 per cent and still widely spread when the value was increased to 0.36 per cent (*viz. m*, Fig. 1). At 0.8 per cent, fairly well defined boundaries were had. With the latter concentration, boundaries in short columns of fluid were found to be displaced a shorter distance from the meniscus than were those in long columns. The same was true of boundaries formed in the same material when run in the electrical centrifuge (*viz. o* and *p*, Fig. 1). Using a very gradual deceleration (2 hours) improved the definition of weak boundaries slightly. As illustrated by *n*, Fig. 1, and *t*, Fig. 2, a considerable improvement was given by the use of a synthetic density gradient.

In the electrical centrifuge, there was no trace of a boundary and no gradation of concentration in long columns with protein concentrations of 0.12, 0.2, and 0.36 per cent (*viz. j*, Fig. 1 and *v*, Fig. 2). There was little, if any, separation of protein from the body of the fluid. With the highest of these concentrations contained in short columns, some clearing of the fluid was noted. The addition of a sucrose gradient to long columns identical to those above made possible the attainment of measurable boundaries in all cases (*viz. k*, Fig. 1, and *w*, Fig. 2). Without the synthetic gradient, concentrations of the order of 0.8 per cent were required to obtain boundaries (*viz. g* and *p*, Fig. 1). Even when the centrifugation time was doubled and the boundary in such a preparation allowed to approach the bottom of the tube, it continued to remain abnormally sharp (*viz. g*). However, the concentration of protein in the supernatant fluid increased (compare *p*). When sucrose gradients were added to long columns of 1.08 per cent protein, the amount of hemocyanin residing in the supernatant fluid decreased considerably (*viz. h* and *i*).

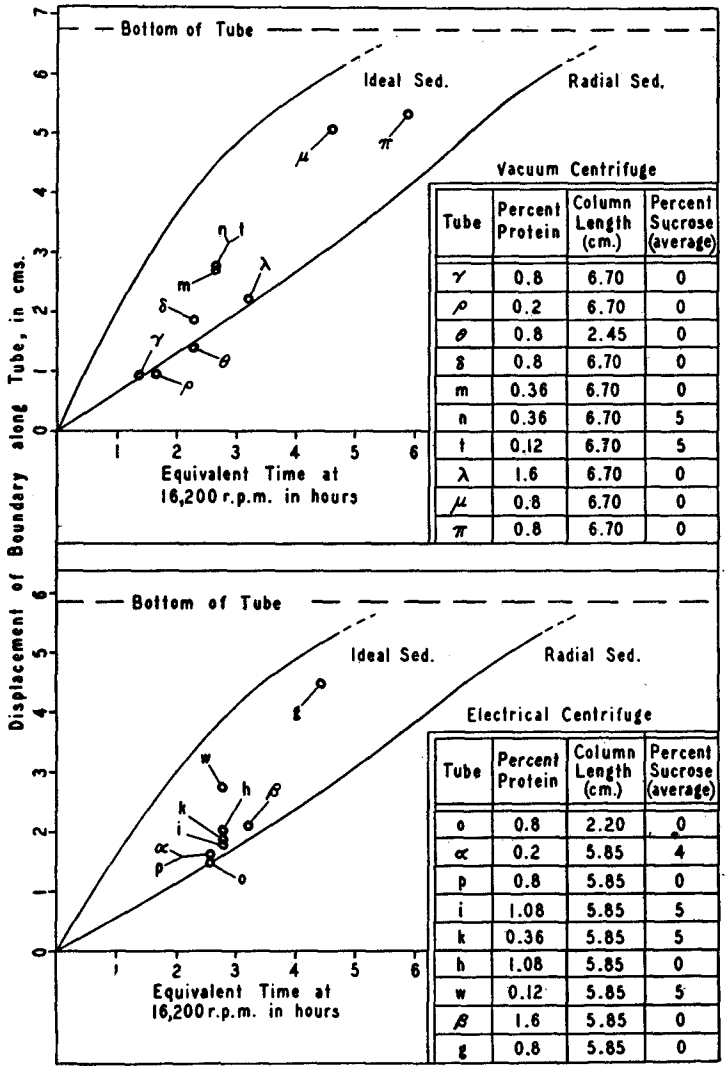
In general, with the electrical centrifuge, boundaries without sucrose were either very sharp or non-existent, depending on the concentration of protein. There was a uniform distribution of protein below the boundary, as well as in the supernatant, where the concentration increased with the centrifugation

time. Obviously, some process was continually active throughout centrifugation maintaining the sharpness of the boundary. In the vacuum centrifuge, boundaries became better defined with increasing concentration, and simultaneously a pronounced gradient in concentration from the boundary to the bottom of the tube became evident. Supernatant fluids were practically free of protein.

In view of the experimental evidence cited, the absence of definition with boundaries of low concentration in the vacuum centrifuge could be explained by disturbances encountered during deceleration and were not necessarily connected with the sedimentation process. With both centrifuges, sucrose gradients caused improvement in every instance. Low concentrations in the vacuum centrifuge were made to behave more nearly like higher concentrations without sucrose. In the electrical centrifuge, the results of the sedimentation process were altered radically and made to resemble more closely those obtained with the vacuum centrifuge. Only a small proportion of the effect could have been accounted for by the slightly increased viscosity of the graded sucrose solutions.

As shown in Text-fig. 1, all boundary displacements which could be measured were plotted against the equivalent time of centrifugation. Because of the low light absorption and for other reasons already cited, values for the lower concentrations can be regarded only as very rough approximations. Within experimental error, the plotted displacements are equal to or greater than those which would have been obtained if the boundaries had progressed away from the axis of rotation at the same rate, measured radially (*radial* curve), as they would have in the analytical ultracentrifuge. The displacements were less than if the boundaries had progressed in a certain ideal manner (*ideal* curve) which will be discussed below.

Some experiments were also carried out in the ultracentrifuge with the cell turned, in a plane perpendicular to the axis of rotation, about 65° from its normal position. In a rough way this arrangement simulated conditions existing in an angle centrifuge. Comparative runs were made with the cell oriented normally, the amount of fluid being so adjusted that the meniscus was situated at the same radial level. Typical results are illustrated by the refractive index photographs of Fig. 3 and the absorption pictures of Fig. 2, which show, respectively, the distribution of concentration gradient and of the concentration itself in paired experiments made under identical conditions. The serial absorption photographs *q* and *r* (Fig. 2) were taken of 0.36 per cent hemocyanin solution at corresponding centrifugation times. Those taken with the tilted cell (*r*) show abnormal and progressive alterations of the concentration, being in the direction of decrease just below the boundary and increase in the lower quarter of the column. Refractive index photographs *C* and *D* (Fig. 3) correspond to the fourth pictures in sets *q* and *r* (Fig. 2), respectively.

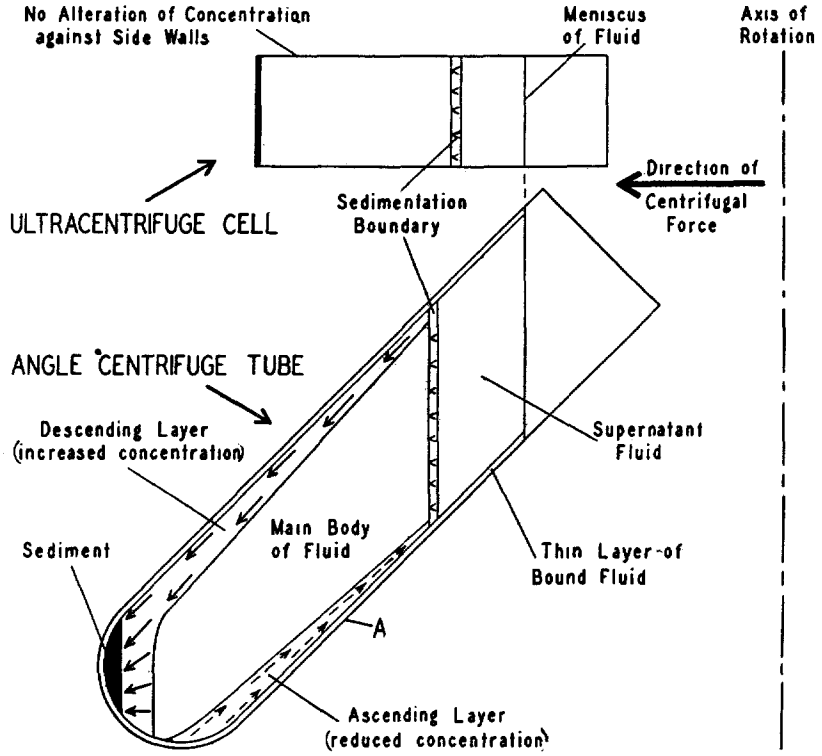


Tube	Percent Protein	Column Length (cm.)	Percent Sucrose (average)
γ	0.8	6.70	0
ρ	0.2	6.70	0
θ	0.8	2.45	0
δ	0.8	6.70	0
m	0.36	6.70	0
n	0.36	6.70	5
t	0.12	6.70	5
λ	1.6	6.70	0
μ	0.8	6.70	0
π	0.8	6.70	0

Tube	Percent Protein	Column Length (cm.)	Percent Sucrose (average)
o	0.8	2.20	0
α	0.2	5.85	4
p	0.8	5.85	0
i	1.08	5.85	5
k	0.36	5.85	5
h	1.08	5.85	0
w	0.12	5.85	5
β	1.6	5.85	0
g	0.8	5.85	0

TEXT-FIG. 1. Experimental results obtained with various concentrations of hemocyanin compared with results which would be obtained if sedimentation took place as in an ultracentrifuge (radial sedimentation curve) or, as would be expected in an ideal case (ideal sedimentation curve) for which sedimentation near the tube wall is fully effective. Synthetic density gradients made with sucrose were used where its presence is indicated.

As the photographs show, the boundary in the misaligned cell was fairly well defined and was displaced only very slightly more in a given time than was the normal boundary. The abnormal concentration gradient introduced between the boundary and the bottom of the cell is clearly evident in *D*. Photographs *A* and *B* are analogous to *C* and *D*, respectively, except that a protein concentration of only 0.04 per cent was employed for the runs. The



TEXT-FIG. 2. Schematic illustration of the sedimentation process in an angle centrifuge as contrasted with that in an ultracentrifuge cell.

behavior of the material in this case was of the same general nature except that the displacement of the boundary in the misaligned cell was significantly greater than in the one correctly oriented. The effect was considerably more pronounced than with higher concentrations and was proportionately greater during the early stages of sedimentation.

Theory of Sedimentation in the Angle Centrifuge.—A general theory which explains the experimentally observed behavior of sedimenting material in the angle centrifuge can be best discussed by reference to Text-fig. 2. Consider first a suspension of an ideal material, one which is homogeneous and non-

diffusible, sedimenting in a centrifuge which is entirely free from convective disturbances. Initially, every particle suffers a displacement proportional to and in direction of the applied centrifugal field at the respective starting point. The particle would continue to move in this direction alone if it were not influenced by other factors. Those particles originally at the meniscus do continue the radial movement and form a sedimenting boundary. The initial radial movement of the particles near the walls causes a clearing of the fluid along the inner surface and a deposition of particles against the outer wall. Because of increasing centrifugal force with radial distance, the thickness of the initial cleared layer and the amount of deposition will be greatest in the bottom sections of the tube. Within the body of the uncleared fluid itself, the divergent movement of the particles causes the concentration to decrease slightly with time, but at a rate which is uniform throughout so that homogeneity continues to exist in this region.

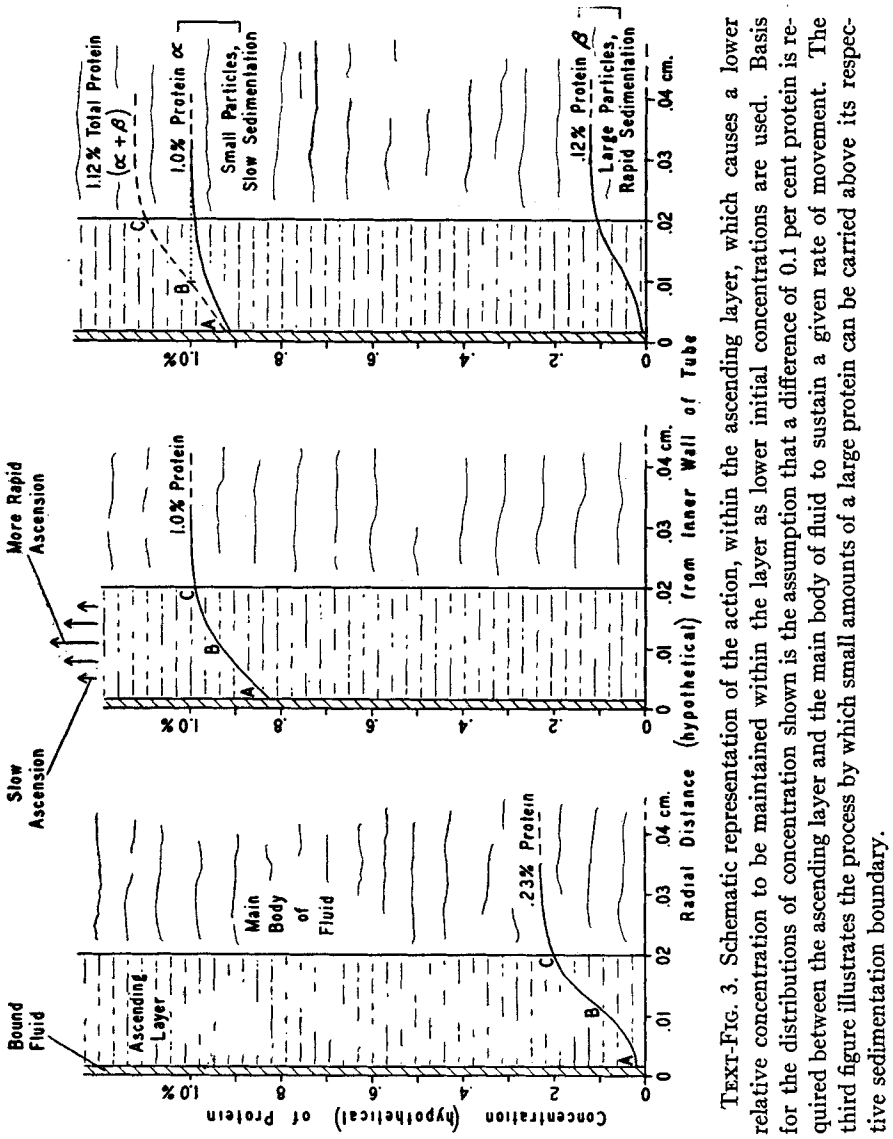
Such a system will continually attempt to adjust itself to the most stable state possible. Any element of fluid which has its density changed from that of its surroundings will seek a new radial level where the density is equal to its own. In accordance with this principle, there is established along the inner wall an ascending layer of clear fluid which rises just above the boundary into the supernatant fluid. The volume of the supernatant fluid is thus increased and the boundary displaced more rapidly than the particles within the boundary actually sediment through the fluid. By a process of integration applied along the inner surface of a given tube for all positions of the boundary, theoretical curves for an ideal material can be constructed which show the progression of a boundary as related to the normal sedimentation of the material in an ultracentrifuge. Curves of this type, based on a normal sedimentation rate equal to that of *Limulus* hemocyanin, have been determined for the full-length columns of fluid used in the present studies. They are represented in Text-fig. 1 as "ideal" curves. Theoretically, the divergence between the radial and ideal curves should increase with increasing tube length and decrease with increasing diameter. Qualitatively, the dependence on column length was verified by experiments which have been described. Up to a certain limit, depending on the dimensions of the tube, the divergence decreases as the angle between the axis of the tube and the radius of the rotor is increased.

After the motion of the ascending stream has become established the layer actually increases in width from the bottom of the tube to the boundary since sedimentation continues as the elements of fluid rise. The movement is opposed only by the viscous drag against the walls and against the body of the fluid. The same applies to the layer of particles deposited against the outside wall. Consequently, the most rapid movement occurs a small distance from the walls, and the body of the fluid below the boundary is made to exhibit a counterclockwise circulating motion by the action of these counteropposite

flows. Immediately adjacent to the wall surfaces may be assumed to exist a very thin layer of bound fluid. Particles deposited within this layer can move along the wall at only an infinitely low rate because of the enormous frictional drag. As the material accumulates the newly arriving particles can move toward the bottom of the tube more easily. In tubes inclined at small angles to the axis of rotation the component of force holding the particles against the wall is very large in comparison with that tending to move them, and it is not surprising that a "sticking" of large particles to the outer wall is frequently observed in such centrifuges. As will be shown below, the effect for real particles subject to Brownian movements is more pronounced in high speed centrifuges than in low speed ones run sufficiently long to give a theoretically equivalent sedimentation.

Ideal sedimentation in the angle centrifuge does not occur with small particles, since they are subject to the forces of diffusion. There is backward diffusion of those particles collecting within the outer layer of bound fluid into the descending layer and from the body of the fluid into the ascending layer. The latter is not cleared completely as with ideal particles but is only diminished somewhat in concentration. Material is not deposited in a semisolid layer that slides down the outer wall but increases the concentration within an adjacent layer of fluid which then descends. Because of diffusion there is a gradient of concentration across the descending layer, and the different portions attempt to settle to levels of corresponding densities within the body of the fluid. At first a gradient of concentration is formed only at the bottom of the cell, but as the process continues each section of the gradient zone is expanded until a good portion of the column's height may be involved. The actual deposition of material takes place only near the bottom of the tube.

As regards the effect on the boundary movement, it can be said that the behavior of large particles in a high centrifugal field will naturally approach that of ideal particles. When the diffusion rate is at all appreciable in comparison to the sedimentation rate, the boundary will be displaced less rapidly. However, the boundary may be expected to progress faster in very dilute solutions than in concentrated ones, as reference to the schematic drawings of Text-fig. 3 will show. These represent the hypothetical distribution of concentration near some point along the inside wall of the tube, such as at A, Text-fig. 2. For the ascending layer to rise at some given average rate, a certain difference in average density is required to be maintained between the layer and the body of the fluid. In the present instance this difference is assumed to be equivalent to a difference in protein concentration of 0.1 per cent. With a low concentration of material (hypothetical 0.23 per cent), considerable migration away from the wall is necessary to establish the required difference. By the time the fluid now at A reaches the region of the boundary, which itself is diffuse, it may be almost cleared of protein and will seek the



TEXT-FIG. 3. Schematic representation of the action, within the ascending layer, which causes a lower relative concentration to be maintained within the layer as lower initial concentrations are used. Basis for the distributions of concentration shown is the assumption that a difference of 0.1 per cent protein is required between the ascending layer and the main body of fluid to sustain a given rate of movement. The third figure illustrates the process by which small amounts of a large protein can be carried above its respective sedimentation boundary.

upper edge of the boundary. The fluid now at C may be of the proper density to seek the central section of the boundary, with that at B rising to an intermediate level. Through the action of the ascending layer the slower portion of the boundary thus becomes more widely spread and the center of the boundary, or region of steepest concentration gradient, suffers a small additional displacement downward.

With a higher concentration (hypothetical 1 per cent), the same density difference (equivalent of 0.1 per cent protein) is reached much sooner, while the concentration at A is still not greatly different, proportionately, from that within the main body of the fluid. The ascending layer rises more rapidly than with the lower concentration, and most of the fluid, especially that now at B and C, seeks levels within the faster section of the diffuse boundary. There is little, if any, additional displacement of the boundary, but the leading edge of the boundary is progressively widened until a fair concentration gradient extends completely to the bottom of the tube.

It is interesting to consider the case in which a material β (Text-fig. 3) of low concentration and high sedimentation rate is centrifuged simultaneously with a smaller, more concentrated protein α. A suspension of virus particles in a solution of serum albumin might furnish such an example. Two sedimentation boundaries will be formed, and if they could proceed according to normal sedimentation, they would in time become well separated with entirely indistinguishable amounts of protein β being found in the vicinity of the diffuse α boundary. However, in the early stages of centrifugation, there is considerable overlapping of the two boundaries by reason of the fact that the rate at which a boundary diffuses or spreads is proportional to the square root of the time, in contrast to a linear relationship for the displacement by sedimentation. Because of the continual removal of partially cleared fluid by the ascending layer, this condition of overlapping is perpetuated, in a sense, along the inner wall of the tube. In the diagram at the right of Text-fig. 3 it can be seen that the levels of comparative density which will be sought by the fluid now between A and B correspond to positions within or near the boundary of protein α. An appreciable quantity of β is carried along by this fluid into regions where it would not otherwise be expected. Of course, if α has an extremely low sedimentation rate, the corresponding ascension to the region of diminished concentration will occur so slowly that almost all of the large, more rapidly moving particles will have time to migrate out of the ascending layer before it has progressed appreciably above the β boundary.

Neglecting the effects of convective disturbances, the imposition of a synthetic density gradient upon a solution or suspension of particles should cause the boundary movement to approach more nearly that exhibited in the ultracentrifuge. As an elemental volume within the ascending layer is partially or completely cleared of sedimentable material, it has to move only a relatively

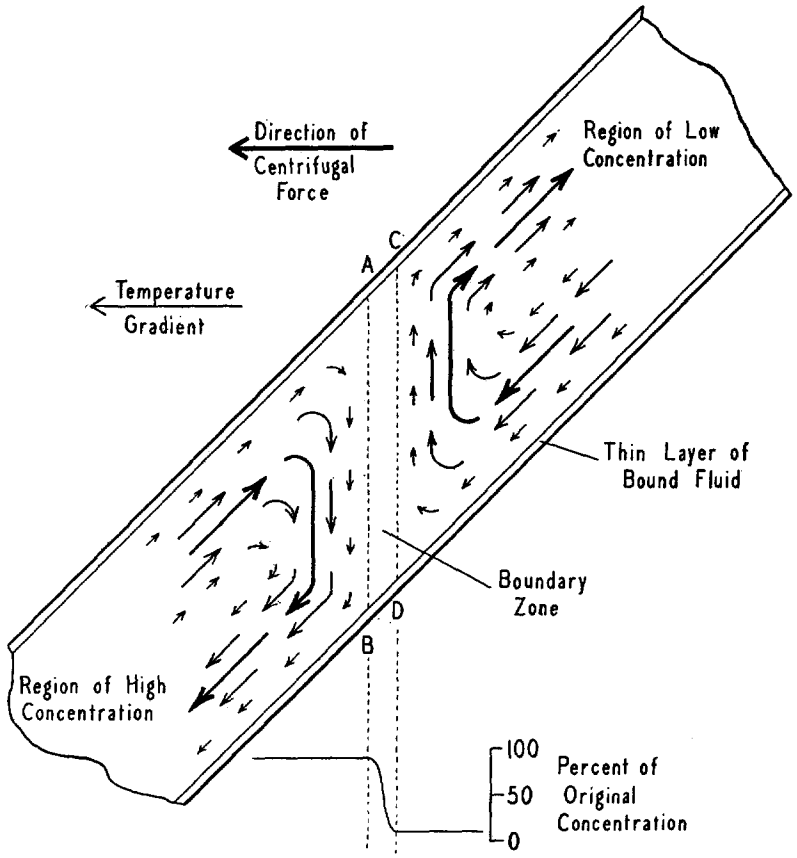
short distance, depending on the strength of the gradient, before it reaches a region of comparable concentration.

Theoretical Considerations of Convective Disturbances.—Even with angle centrifuges of the vacuum type, in which conditions are ideal as regards the absence of temperature disturbances, some remixing of partially sedimented material occurs during deceleration, as was shown experimentally. This is caused by the tendency of the fluid to continue the rotation. The liquid near the outer walls is decelerated at a more rapid rate, in linear measure, than that closer to the axis of rotation. Across the fluid along the radius exists a gradient of tangential decelerating forces which causes a torque to be exerted on the fluid. Thus a circulation is established in the same direction as the direction of the centrifuge's rotation. The viscous drag against the walls helps to slow down the circulation, but only an appropriate density gradient establishing a countering torque can prevent it. This torque is due to the tendency of the denser elements to remain near the outer wall of the tube. The magnitude of these forces which are opposed to the circulation is directly proportional to the centrifugal force, whereas the forces prompting circulation are directly proportional to the rate of deceleration. Hence it is important to decelerate a centrifuge especially slowly just before it stops, when the centrifugal force is very small. The action of a synthetic density gradient in minimizing stirring is immediately apparent. Incidentally, the gradient due to hydrostatic compression of the fluid offers appreciable assistance in reducing convective disturbances only in very high centrifugal fields (13). It can be shown that even then the only type of convection which can be completely inhibited is that which would otherwise be caused by a temperature gradient directed exactly parallel to the centrifugal field.

A centrifuge such as the electrically driven one (5) used in the present studies which spins in the open air or in any other gas is subjected to the frictional resistance of the gas, causing a generation of heat which must be dissipated into the same medium. The surface consequently tends to warm up to a certain equilibrium temperature which is higher for a more rapidly moving surface. As a result heat will be transferred from the outer to the slower moving inner sections of a rotor, and a temperature gradient toward the outer sections will be maintained.

Referring to Text-fig. 4, which represents a section of a centrifuge tube, consider as a simplification that the temperature gradient is parallel to the centrifugal force. An elemental volume of fluid in the outer portions of the tube, for example, is thus warmer and consequently less dense than the neighboring fluid located closer to the axis of rotation. This elemental volume tends to seek a new level nearer the axis of rotation where the density of the fluid is more comparable to its own. This tendency can be quite appreciable even with very small temperature gradients, since the buoyancy forces increase

directly with the value of the centrifugal force. The attempt of the fluid to reach a more stable state generally results in a circulating flow or convection. In the case of a water-filled ultracentrifuge cell with an imposed radial temperature gradient, the fluid would rise along the side walls and return with lowered



TEXT-FIG. 4. Section of an inclined centrifuge tube illustrating how, during operation of an angle centrifuge spinning in the open air, convection currents above and below the boundary keep it abnormally sharp.

temperature down the central portion of the cell. With an inclined cylindrical tube containing fluid in which a sedimenting boundary is present it is difficult to predict either the exact flow pattern or the distribution of temperature throughout the fluid although the temperature variation along the walls be known. However, the schematic drawing of Text-fig. 4 may be used for purposes of argument.

Across a sedimenting boundary there is a comparatively rapid change of

fluid density with radial distance by reason of the gradient of solute concentration. Below the boundary, for example, any elemental volume of fluid seeking a new level of slightly different fluid density will find it necessary to rise only to the outer edge of the boundary if the concentration of sedimenting material is sufficiently high. It can be seen, as indicated in the drawing, that separate circulating flows are set up above and below the boundary. Whenever the concentration gradient at either edge of the boundary reaches a certain minimum value, *i.e.* when the difference between the density of circulating fluid near the boundary and the density of an adjacent layer within the boundary reaches a certain minimum value, the respective layer becomes incorporated in the circulation. Thus the boundary is kept abnormally sharp. The steep concentration gradients within the boundary orient themselves so as to furnish a counter torque which prevents stirring that otherwise would be initiated within the boundary through the action of temperature gradients and shearing forces. Since there is a steep gradient of concentration through the boundary region, there will be a continuous and relatively rapid diffusion of particles across the boundary into the less concentrated region. The convection currents above and below the boundary maintain uniform distributions of concentration in these regions.

Within the boundary zone the radial sedimentation of particles proceeds normally, and thus the boundary zone, the zone of high concentration gradient, is made to progress along the tube. However, its rate will not be characteristic of a normal radial sedimentation because of factors related to the accelerated diffusion across the boundary. The activity or strength of the circulating flow on either side of the boundary is determined in considerable degree by the height of the respective column of fluid. Because of the restraining action of the bounding surfaces and the increased interference offered by the counterflow, convection is reduced in short or narrow columns. For example, when a boundary reaches the lower section of a tube, the convection is more active above and less active below. Particles then diffusing across AB (Text-fig. 4) into the boundary zone are not so readily replaced on the left of AB by the circulation, and the concentration in the vicinity of AB is decreased. The influence of this decrease is transmitted through the boundary, lowering the concentration at every point. When the gradient at CD is decreased below the threshold value by lowering of concentration within the boundary zone, the fluid there (near CD) is then able to join the circulating flow above the boundary. With this process in continuous operation the net result is an additional progressive displacement of the boundary along the tube. By a similar argument it can be shown that theoretically, as was actually observed experimentally, a boundary near the meniscus can be displaced toward the meniscus. However, in actual centrifugations this action appears to be subdued since observed rates have always been greater than the value for normal,

radial sedimentation. This is possibly explained by the fact that whereas convection in the supernatant is comparatively uninhibited, interference below the boundary is offered by the directly opposing motion of the ascending and descending layers established by the sedimentation of particles close to the walls.

A certain gradient of concentration must be maintained within the boundary zone to prevent a single circulatory system from being established for the whole column. With a very low concentration of material, the distance between *AB* and *CD*, *i.e.*, the width of the boundary zone, must then become very small. Since the gradient is kept at a minimum value, the same number of particles can be transported per second as with higher concentrations, but, because of the smaller total number of particles concerned, equal concentrations will be quickly established on both sides of the boundary, and the boundary will vanish.

Where thermal convection is present, with or without boundary formation, sedimentation continues to take place but is theoretically never complete. The more pronounced the convection and the lower the concentration of material, the slower the deposition of sediment at the bottom of the tube. Particles migrate toward the layer of bound fluid along the outer wall, and those which do not diffuse back into the circulating fluid form aggregates or a denser layer of solution which is able eventually to reach the bottom of the tube. As a first approximation sedimentation will be more or less logarithmic; during any given interval of time a certain fixed proportion of the particles still circulating within the fluid at that time will be deposited. Concentration gradients are so steep in the vicinity of the sediment that little remixing into the fluid occurs.

Thermal convection can be prevented by imposing on the solution a synthetic density gradient of some non-sedimentable material. The strength of the gradient should be sufficient to counteract completely the opposing density gradient set up by the variation of temperature through the fluid.

Discussion of Practical Aspects.—From an investigation of a given material with an angle centrifuge, it is hazardous to draw any conclusions regarding normal sedimentation rate, particle size, or homogeneity unless the existence of a sedimentation boundary has been demonstrated. For small tubes (1.5 cm. diameter or less) in a properly operated centrifuge of the vacuum type, a very approximate semiempirical relationship could be derived for determining the amount of sediment in terms of normal sedimentation rate, provided the concentration of material were known to exceed a few tenths of 1 per cent. However, a method based on measuring the displacement of a boundary has certain important advantages and is more general in its application. It has been shown that tubes can be removed from a centrifuge, reoriented, and sampled or photographed without greatly altering a boundary. Stirring during deceleration is more serious but can be rendered inconsequential by decelerating slowly just before stopping the centrifuge and by providing a sufficient opposing

density gradient, either synthetically or by the use of relatively concentrated material.

From consideration of both experiment and theory, it can be said that the rate (measured radially) at which a boundary will migrate away from the axis of rotation in an angle centrifuge of the vacuum type is equal to or somewhat greater than the normal rate as measured in an ultracentrifuge. For tubes of ordinary dimensions the actual rate should never exceed the normal rate by a factor of as much as 2. The same generalities apply to centrifuges spinning in the open air, but they must be regarded with less certainty because of the thermal convection currents of unpredictable nature which have been experimentally demonstrated in such cases. For efficient separation in centrifuges of the latter type, tubes of the smallest practical bore should be used, although some compromise with capacity may be necessary.

A boundary, *i.e.*, the region where there is a relatively abrupt change in the concentration, may be located by optical means or by other tests made on samples taken carefully from the tube at different levels (3). A supernatant fluid relatively free of the material under study and a marked gradient of concentration between the boundary and the bottom of the tube are characteristic of optimum sedimentation in an angle centrifuge. However, there is ample physical basis to explain the presence of small amounts of a homogeneous material in the supernatant fluid. Although it may be impossible to reach definite conclusions regarding the relative particle size of such residual material, inferences perhaps can be drawn if results are compared with new determinations made after incorporating all the refinements which have been discussed

If thermal convection has been very active, a uniform distribution of material will be found above the boundary and also in a higher concentration below the boundary. The boundary may be abnormally sharp. Incidentally, phenomena studied in the present work can be used to test, by a simple procedure, for possible thermal convection in the ultracentrifuge cell (10). In the first place, unusually sharp boundaries should always be regarded with suspicion. If a slight misalignment of the cell from its normal position does not cause an additional concentration gradient to be introduced below the boundary, it may be concluded that convection is present.

In designing angle centrifuges for purely preparative purposes one is usually interested in attaining the maximum possible efficiency, which may be defined as the ratio between the volume of fluid cleared and the equivalent time required at speed. Allowances are made for the time of acceleration and deceleration in figuring the equivalent time. As an approximation, the efficiency may be considered as proportional to the product of the average centrifugal force, the total capacity of the centrifuge, and the reciprocal of the projected distance, measured radially, between the surface of the fluid and the outer edge of the tubes. Greatest efficiency with diffusible material can be realized with tubes oriented at small angles (10–20°) to the axis of rotation. Centri-

fuges with such tubes have been described by Masket (11), and a theoretical treatment of the stresses developed has shown them capable of high speeds in spite of large numbers of tube holes (12). However, small angles are not suitable for general clarification work with large non-diffusing particles, which will collect along the wall of the tube. Furthermore, special arrangements (11) to prevent overflow must be provided, or else space is wasted and tubes collapse easily; also the precision with which a boundary can be located is probably lower with small angles.

When a centrifuge is to be employed for general purposes, including rough analysis or rapid clarification, angles near 35° and tubes of 1.3 cm. bore have been found to represent a satisfactory compromise between efficiency and other factors. If particles of molecular weight below 100,000 are to be studied, centrifugal forces of at least 200,000 gravity should be employed in order that the boundary may clear the meniscus within a reasonable time (3, 12).

Convective disturbances of all types can be almost completely eliminated by imposing upon the liquid under study a synthetic gradient of sucrose or some other material of low molecular weight. In the present investigation, the gradients were prepared in a very rough uncertain manner and perhaps inadequately in some cases. It is believed that a great improvement might be had by preparing several samples of the material, each incorporating a few per cent more of sucrose, for example. These could be introduced into the tube at the bottom, starting with the least dense, and the tube allowed to stand for a time to permit a relatively uniform gradient to be established by diffusion. With a well formed and sufficiently steep gradient the boundary of a homogeneous material should progress at very nearly the normal (ultracentrifugal) rate, measured radially. Of course, the increased viscosity of the fluid over the section traveled must be taken into account and can be determined by measurements upon samples taken after the run. Furthermore, the technique can be applied to preparative procedures which have produced unsatisfactory concentration of a particular biological agent, for example.

Where convection has been minimized the following approximate formula can be used either for roughly estimating the particle size, assuming no extreme deviation from a spherical shape, or for approximating the time required to complete a sedimentation of nearly spherical particles whose physical properties are known:

$$T = 54\left(\frac{D - L}{D + L}\right)\left(\frac{\eta}{d^2(\sigma - \rho)S^2}\right)$$

where S is the rotational speed in R.P.M.; L is the radial distance in centimeters from the meniscus to the axis of rotation; D is the radial distance from the boundary (or outer edge of tube for complete sedimentation) to the axis; T is the time in minutes; ρ and σ are the densities in grams per cubic centimeter of the medium and the particles, respectively; η is the viscosity of the fluid in

poises; d is the average diameter of the particle in centimeters. If θ is the angle between the tube and the axis of rotation, then for a boundary displaced X cm. along the tube from the miniscus, $D = X \sin \theta$. As an approximation of average conditions with aqueous solutions the formula can be reduced to:

$$T = \frac{1.8}{d^2 S^2}\left(\frac{D - L}{D + L}\right)$$

where the viscosity has been taken as 0.01 poise and the fluid and particle densities as 1 and 1.3 gm. per cc., respectively.

SUMMARY

1. Using hemocyanin from *Limulus polyphemus* as a test material, the process of sedimentation in the angle centrifuge, operating both in vacuum and in the open air, has been investigated.

2. Sedimentation in a given field of force was found less efficient when centrifugation was conducted in the open air, because of thermal convection.

3. Correlations have been made with results obtained in the analytical ultracentrifuge, and a theory of sedimentation in inclined tubes has been presented to explain the experimental results.

4. It has been shown that under proper conditions the angle centrifuge may be used for approximate determinations of particle size.

5. Recommendations, based mostly on experimental evidence, have been made for improving sedimentation and interpreting results.

6. To counteract convective disturbances of either thermal or inertial origin, a satisfactory method has been developed which consists of furnishing the fluid under study with a synthetic density gradient, formed with sucrose or some other non-sedimentable material.

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EXPLANATION OF PLATES

PLATE 1

FIG. 1. Pictures *a* to *f* illustrate the effect on artificial boundaries of *Limulus* hemocyanin of acceleration to and immediate deceleration from 16,200 R.P.M. All boundaries were made to the same level as that of unspun sample *a* and allowed to stand for 135 minutes. Pictures *g* to *p* show the results of centrifugation at the above speed in angle centrifuges, except for *l*, which was obtained with the analytical ultracentrifuge. Equivalent centrifuge time: *h* to *p*, 135 minutes; *g*, 270 minutes. Vacuum centrifuge: *c, f, m, n;* others in open-air centrifuge. Concentration of 0.2 per cent, *a, b, c;* 0.8 per cent, *d, e, f, g, o, p;* 0.36 per cent, *j, k, l, m, n;* 1.8 per cent *h* and *i*. Pictures *i*, *k*, and *n* illustrate improvement of boundaries by addition of synthetic density gradient. In the photographic negatives the boundary of *o* appears blurred with the average position close to level indicated.

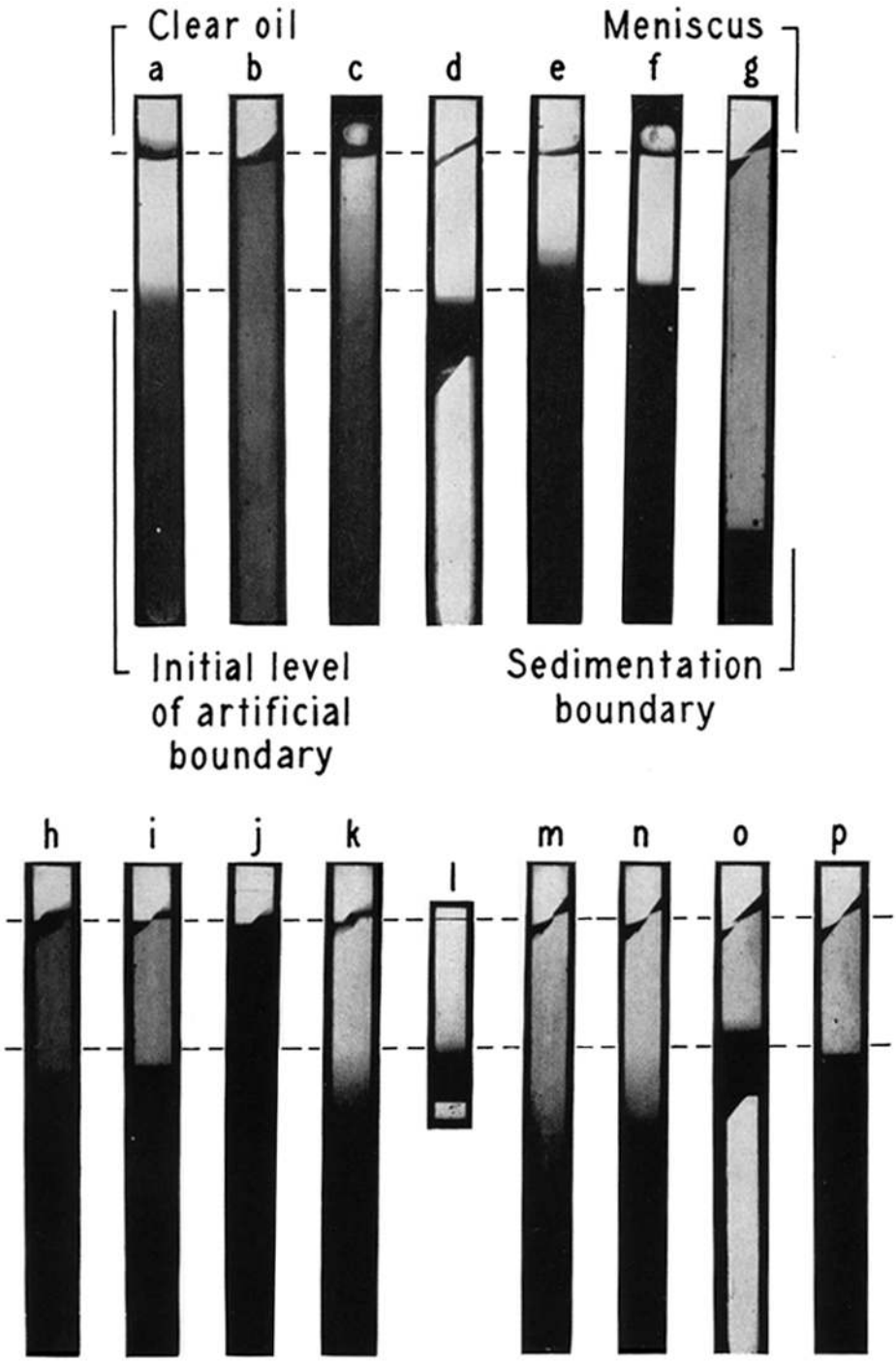

FIG. 1

(Pickels: Sedimentation in the angle centrifuge)

PLATE 2

FIG. 2. Serial absorption photographs (*q* and *r*) comparing the sedimentation of 0.36 per cent hemocyanin in a normally oriented ultracentrifuge cell (*q*) with that in the same cell after a 65° misalignment (*r*). Pictures *s* to *w* represent experiments similar to those of Fig. 1, except that the concentration is only 0.12 per cent. Vacuum centrifuge, *s* and *t*; open-air type, *v* and *w*. Synthetic density gradient of sucrose was provided in *t* and *w*.

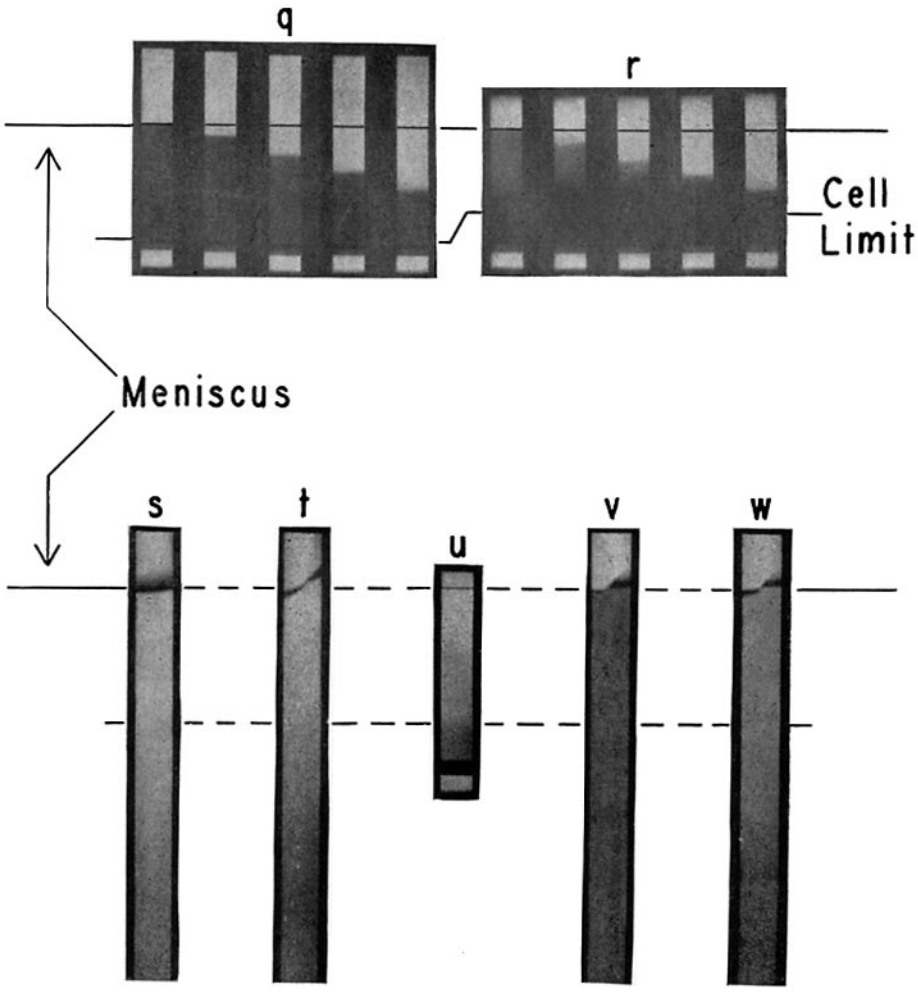

FIG. 2

PLATE 3

FIG. 3. Photographs taken by a refractive index method and showing sedimentation at corresponding times in normal (*A*, *C*) and misaligned (*B*, *D*) ultracentrifuge cells. *C* and *D* correspond to the fourth pictures in sets *q* and *r*, respectively, of Fig. 2. The concentration of hemocyanin in cases *A* and *B* was only 0.04 per cent.

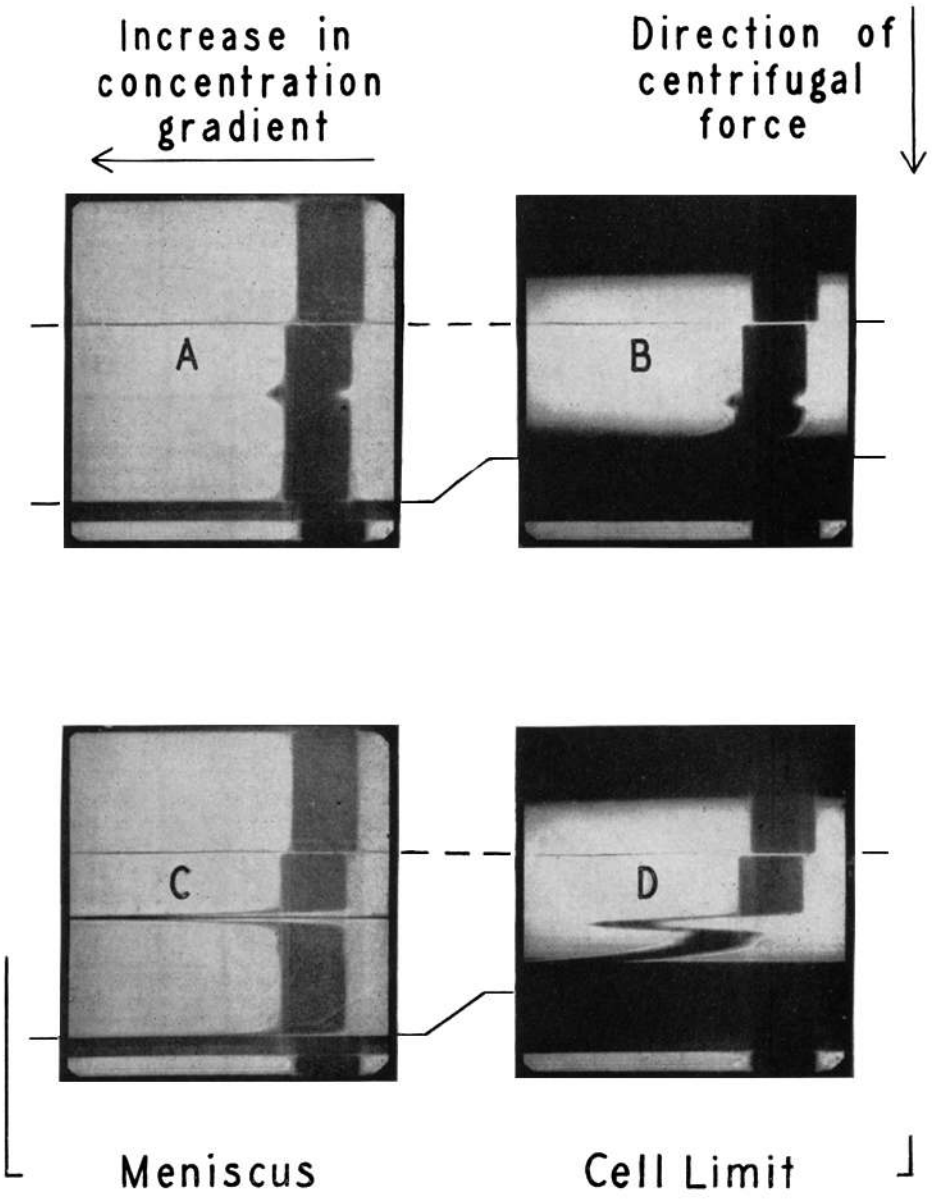

FIG. 3

(Pickels: Sedimentation in the angle centrifuge)